UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA VEGETAL

Embryonic thymic epithelium differentiation in chicken: study of molecular signals involved in lymphoid progenitor cells colonization

Carlota Bobone Lucena

DISSERTAÇÃO

MESTRADO EM BIOLOGIA MOLECULAR E GENÉTICA

2012

UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA VEGETAL

Embryonic thymic epithelium differentiation in chicken: study of molecular signals involved in lymphoid progenitor cells colonization

Dissertação orientada por
Professora Dra. Hélia Cristina de Oliveira Neves (FMUL)
Professora Dra. Rita Maria Pulido Garcia Zilhão (FCUL)

Carlota Bobone Lucena

MESTRADO EM BIOLOGIA MOLECULAR E GENÉTICA

2012

Agradecimentos

À Hélia, por me ter recebido no seu laboratório e pela dedicação e preocupação que sempre mostrou comigo e com o meu trabalho; pela autonomia e liberdade me proporcionou e por tudo o que aprendi durante este ano.

À Professora Rita, pela oportunidade de a ter como orientadora, pela disponibilidade e apoio que sempre demonstrou e sobretudo pela contribuição para o meu gosto pela investigação desde os tempos da licenciatura.

À Unidade de Biologia da Hematopoiese do Instituto de Histologia e Biologia do Desenvolvimento da FMUL, pelo acolhimento e ajuda na realização desta tese. Em especial à Isabel pelo exemplo de boa vontade, pelos conselhos, e especialmente pela ajuda essencial para a análise de citometria, e ao Vítor, pela sua dedicação e disponibilidade, por toda a ajuda sem a qual o trabalho não estaria completo.

À Marta pelo dia-a-dia no laboratório, pelos grandes momentos (em tempo e qualidade) que passámos juntas, pela sua boa disposição contagiante, e "espírito de equipa": essencialmente pela amizade que se criou.

A todos os que passaram pelo laboratório, especialmente ao Rafael, à Marta e mais recentemente à Joana, pela ajuda indispensável para a realização deste trabalho.

À minha família e aos meus amigos, pela presença constante e apoio em todos os momentos, pelo exemplo que são e que me estimula a ser sempre mais.

Obrigada!

Resumo

O timo é o órgão linfóide primário responsável pela diferenciação de progenitores hematopoiéticos linfóides (PHL) em linfócitos T maduros. Após o nascimento, os PHL entram no timo através dos vasos sanguíneos e iniciam a timopoiese, processo complexo de diferenciação em linfócitos T. Os PHL começam por especificar-se na linhagem T e depois iniciam o seu longo processo de diferenciação adquirindo coreceptores de membrana característicos das células T maduras, como os marcadores CD3, CD4 e CD8. A timopoiese é essencial para a constituição de um sistema imunitário saudável. De facto, os linfócitos T são o componente principal do sistema imunitário adaptativo, capazes de responder a agentes infecciosos, e aumentando a capacidade de defesa do organismo com cada exposição a antigénios específicos.

O timo é um órgão constituído por células epiteliais tímicas (CET), células mesênquimais derivadas da crista neural (CN), células dos vasos sanguíneos e células hematopoiéticas. O seu desenvolvimento está intimamente ligado ao das glândulas paratiróides, uma vez que os seus epitélios partilham a mesma origem embrionária: a endoderme das terceira e quarta bolsas faríngicas (3/4 BF)[1,2]. A origem endodérmica das células epiteliais foi pela primeira vez demonstrada por Le Douarin e Jotereau utilizando o modelo de quimeras galinha-codorniz[3]. Neste trabalho também mostraram que distintos mesênquimas ectópicos, são capazes de suportar (mesênquima permissivo, da somatopleura) ou não (mesênquima não permissivo, do sómito ou do botão do membro) o desenvolvimento da endoderme na formação dum timo funcional, revelando a importância das interações celulares entre a endoderme e o mesênquima adjacente, nas fases iniciais do desenvolvimento tímico[3]. Durante esta primeira fase do desenvolvimento tímico, ocorre a especificação das CET[2].

Os territórios presuntivos do timo e das glândulas paratiróides são definidos pela expressão de factores de transcrição distintos nas bolsas faríngicas, o gene *Foxn1* (*forkhead box N1*) e o gene *Gcm2* (*glial cells missing-2*), respectivamente[4]. Assim, em galinha, o rudimento do timo foi identificado ao dia 4,5 de desenvolvimento embrionário (E4,5) (E4 em codorniz) na região dorsal das 3/4 BF enquanto que o domínio das paratiróides ocupa um território mais anterior e ventral das mesmas[5].

A segunda fase do desenvolvimento do timo depende da sua colonização por PHL e de interações entre estes e as células epiteliais tímicas. Estas interações são essenciais para a diferenciação das CET em duas linhagens celulares, cortical e medular, originando consequentemente a formação de dois compartimentos tímicos: o córtex e a medula[6–9]. Durante a embriogénese, Le Douarin descreveu três ondas de colonização do epitélio tímico (ET) por PHL, no modelo de galinha[10]. A primeira onda ocorre ao E6,5 (E6 em codorniz),

antes da vascularização do rudimento tímico, entrando os progenitores hematopoiéticos no rudimento tímico através da sua migração pelo mesênquima adjacente[3,11,12]. As restantes ondas realizam-se através dos vasos sanguíneos, ao E12 e E18[10].

As interações entre os PHL e as TEC são mediadas por vários factores solúveis, como citocinas (estimulam a proliferação dos timócitos) e quimiocinas (importantes na migração dos PHL para o rudimento tímico), e por vias de sinalização como a sinalização Notch. A importância do microambiente Notch para a correcta especificação de progenitores hematopoiéticos nas diferentes linhagens linfóides foi demonstrada pela primeira vez em 2001 pelo grupo de L. Parreira[13]. Além disso, os genes envolvidos na sinalização Notch (receptores, ligandos e genes-alvo) são expressos de forma distinta nos diferentes territórios do timo adulto, reforçando a importância desta via de sinalização na função do mesmo[14]. Recentemente, o grupo de H. Neves observou em embriões de galinha que os genes envolvidos na sinalização Notch estão diferencialmente expressos na endoderme das bolsas faríngicas, em estádios prévios à formação do rudimento tímico.

Em estudos recentes, este mesmo grupo, utilizando o modelo de quimeras galinha-codorniz, observou que células da endoderme das 3/4 BF de codorniz (dador; E3) quando enxertadas num ambiente permissivo (mesênquima da somatopleura) dum embrião de galinha (receptor; E2,5), especificam em ET (*Foxn1*$^{+}$). Também constataram que, 5 dias após o enxerto, o epitélio tímico se encontra colonizado por PHL com origem no embrião receptor (galinha)[5]. Contudo, quando a endoderme é enxertada num ambiente não permissivo (mesênquima do botão embrionário do membro posterior), nas mesmas janelas temporais, esta expressa *Foxn1* mas não é colonizada por células hematopoiéticas[5]. Estes resultados sugerem que, quando enxertada num ambiente não permissivo, a endoderme não possui e/ou não recebe os sinais do mesmo (mesênquima adjacente) necessários à sua colonização por PHL. De facto, em codorniz, a primeira vaga de colonização do rudimento tímico por PHL (E6) ocorre durante a janela temporal em análise. Assim, este sistema representa um novo modelo de estudo para identificar os factores envolvidos na migração dos PHL e na colonização do rudimento tímico pelos mesmos, etapas fundamentais ao desenvolvimento do timo.

Este trabalho teve como primeiro objectivo identificar os sinais moleculares da via de sinalização Notch envolvidos na fase de colonização do rudimento tímico (CET) pelos PHL. Para este efeito utilizou-se o modelo de quimeras galinha-codorniz acima descrito. Embriões quiméricos, com 3 e 5 dias de desenvolvimento após o enxerto da endoderme, foram sacrificados e estudados por hibridação *in situ* com genes envolvidos na sinalização Notch, especificamente os ligandos-Notch, *Delta1* e *Delta4*. Os resultados obtidos mostram que a endoderme, quando enxertada no ambiente permissivo, expressa ambos os ligandos, nos

dias 3 e 5 após o enxerto. Em contraste, quando a endoderme é enxertada num ambiente não permissivo, embora expresse *Delta4*, apresenta uma expressão diminuída ou inexistente de *Delta1* (3 e 5 dias após o enxerto). Assim, os resultados sugerem que *Delta1* poderá ser o ligando Notch específico envolvido na mediação do sinal Notch na interação entre o epitélio (ligando Delta1) e os PHL (receptor Notch) nas fases iniciais de colonização do epitélio tímico. Em conformidade, no timo adulto de ratinho foi descrita a expressão de *Delta1* na junção cortico-medular, local de entrada dos PHL após o nascimento[14].

Atualmente, decorrem estudos para aprofundar o papel deste ligando e caracterizar a sua sinalização durante o processo de colonização do epitélio tímico. De futuro, também serão estudadas outras moléculas possivelmente envolvidas neste processo, tais como quimiocinas, e componentes de outras vias de sinalização.

Em paralelo, neste trabalho caracterizou-se o desenvolvimento do timo e das glândulas paratiróides em galinha (entre os dias 5 e 18 de desenvolvimento), ao nível histológico e ao nível da diferenciação celular dos linfócitos T no rudimento tímico (por análise de citometria de fluxo).

A análise histológica foi realizada através da expressão *in situ* dos genes *Foxn1* e *Gcm2*. Em cortes seriados de parafina, ao longo dos estádios estudados (E5 a E13), observou-se a expressão de *Foxn1* e *Gcm2* nos rudimentos do timo e das glândulas paratiróides, respectivamente. Concluiu-se que a expressão destes factores de transcrição é mantida até ao dia E13. Mostrou-se também que as glândulas paratiróides apresentam uma morfologia adulta a partir de E9, e que o timo ao dia 13 de desenvolvimento se encontra ao longo do pescoço e diferenciado em dois compartimentos (córtex e medula).

Analisou-se a dinâmica de diferenciação das populações de timócitos presentes no rudimento tímico, entre E11 e E18, por citometria de fluxo, utilizando os marcadores de membrana CD3, CD4 e CD8. Os resultados desta análise mostram que até E13 existem duas populações imaturas de timócitos, $CD8^{+}CD4^{-}CD3^{-}$ e $CD3^{+}CD8^{-}CD4^{-}$, pouco proliferativas, e derivadas da primeira onda de colonização, sugerindo que podem ter um papel na especificação do epitélio tímico. De facto, no momento da segunda onda de colonização (E12-13) a medula começa a ser definida, evidência morfológica do processo prévio de especificação das células epiteliais em linhagens cortical e medular. Paralelamente, após a segunda onda de colonização (>E15) observa-se uma forte expansão de células duplas positivas, $CD4^{+}CD8^{+}$, sugerindo que estes timócitos têm uma maior capacidade de se diferenciarem (e expandirem) e possivelmente de contribuir para a maturação dos dois compartimentos tímicos.

Palavras-chave: organogénese do timo, epitélio tímico, colonização por progenitores hematopoieticos linfóides, *Foxn1*, *Delta1*.

Abstract

The thymus is the primary lymphoid organ where maturation of lymphoid progenitors cells (LPCs) into T-cell occurs. This maturation depends on interactions between the LPCs and thymic epithelium (TE). TE derives from endoderm of the 3rd and 4th Pharyngeal Pouches (3/4 PPs) (in chicken) and depends on epithelial-mesenchymal interactions and on LPCs colonization to become functional.

Using the quail-chick model, our group showed that distinct mesenchymal tissues are permissive (somatopleure) or non-permissive (limb bud) to 3/4 PPs endoderm specification and early development. In addition, only in the permissive environment the endoderm is colonized by LPCs.

In this work, we aimed to identify the molecular cues, namely Notch signaling ligands, involved in TE colonization by LPCs. For that, we used the quail-chick chimeric model. Using immunohistochemistry combined with *in situ* hybridization techniques we showed that *Delta1*, as opposed to *Delta4*, is down-regulated in the endoderm developed in the non-permissive mesenchyme (with no LPCs colonization). Together, these results suggest that mesenchymal-epithelial interactions are important to establish a proper environment to thymus formation and that Delta1 is involved in TE colonization by LPCs.

In parallel, we described thymus and parathyroid glands development through the *in situ* expression analysis of specific transcription factors for each organ (*Foxn1* and *Gcm2,* respectively). We observed that the expression of both transcription factors is maintained from E5 to E13 in the respective developing organs.

We then characterized thymocyte populations during thymic organogenesis by flow cytometry analysis. We identified two immature populations, single positive for CD3 or CD8, at early thymic development (<E13), suggesting that these thymocytes may be important in TECs specification into different lineages (cortical and medullary), and subsequent maturation of thymic compartments.

With this work, we hope to clarify important events in thymus organogenesis: colonization by LPCs and TECs specification.

Keywords: thymus organogenesis, thymic epithelium, lymphoid progenitors cells colonization, *Foxn1*, *Delta1*.

Index

Abbreviations

APC – Antigen Presenting Cells

DC – Dendritic Cells

DMEM – Dulbecco's Modified Eagle Medium

DN – Double Negative

DNA – Deoxyribonucleic Acid

DP – Double Positive

E – Embryonic day

EDTA – Ethylenediaminetetraacetic Acid

FACS – Fluorescence Activated Cell Sorter

FBS – Fetal Bovine Serum

h, min, sec, ms – hour, minute, second, millisecond

HE – Hematoxylin-Eosin

IHC – Immunohistochemistry

ISH – *In Situ* Hybridization

LPC – Lymphoid Progenitor Cell

MHC – Major Histocompatibility Complex

NC – Neural Crest

NS – Negative Selection

ON – overnight

PA – Pharyngeal Arch

PBS – Phosphate Buffered Saline

Pen/Strep – Penicillin/Streptomycin

PFA – Paraformaldehyde

PP – Pharyngeal Pouch

PS – Positive selection

PTE – Parathyroid Epithelium

PTH – Parathyroid Hormone

RNA – Ribonucleic Acid

RT-PCR – Reverse Transcriptase-Polymerase Chain Reaction

SP – Single Positive

TCR – T-cell Receptor

TE – Thymic Epithelium

TEC – Thymic Epithelial Cell

TN – Triple Negative

Introduction

I. The Thymus: Structure and Function

The thymus is the primary lymphoid organ that supports the differentiation of Lymphoid Progenitor Cells (LPCs) along the T-cell pathway. T-cells are the main component of the adaptive immune system, capable to respond to infectious agents and to increase in magnitude and defensive capabilities with each successive exposure to antigens.

In mammals, the thymus is a bilobed organ, located in the central compartment of the thoracic cavity, above the heart and behind the sternum. In birds two thymi exist, located bilaterally near the jugular vein, along the neck, subdivided in seven lobes each (Figure 1), divided themselves in lobules[1,5]. In mammals, located in the vicinity of the thymus, there are four parathyroid glands. In birds, there are also four glands located under the thyroid glands, near the nodose ganglion and the ultimobranchial body[5] (Figure 1). These glands are small endocrine glands that produce the parathyroid hormone (PTH), regulating calcium and phosphate homeostasis in the organism[15].

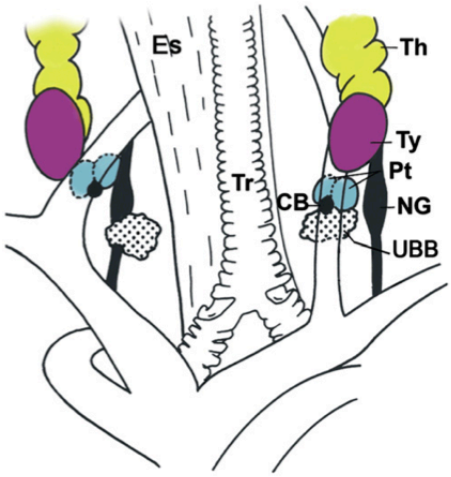

Figure 1. **Schematic representation of the anatomic location of foregut endoderm-derived glands in adult chicken.** (CB, carotid body; Es, esophagus; NG, nodose ganglion; Pt, parathyroid glands; Th, thymus; Tr, trachea; Ty, thyroid; UBB, ultimobranchial body). (Adapted from Neves et al. 2012[5])

The thymus is an epithelial organ surrounded by a capsule, consisting of a thick layer of connective tissue, and each lobule consists of an outer cortex and an inner medulla. The cortex and medulla are composed by distinct and specific Thymic Epithelial Cells (TECs), blood vessels cells, bone marrow derived cells (mostly macrophages and dendritic cells) and mesenchymal cells. Cortical-TECs (cTECs) and medullar-TECs (mTECs) form a three-

dimensional network densely packed. Together these elements form the stromal component of the thymus that supports the maturation of LPCs into T-cells.

In the mature thymus, developing T-lymphocytes (called thymocytes) make up more than 95% of its cellularity[11], where the cortex contains a dense population whereas the medulla is more sparsely populated. With age, the thymus involutes (invasion of the parenchyma by adipose tissue) and is virtually undetectable in post-pubertal humans[16]. In chicken, the size and weight of the thymus reach their maximum at the second week of life, and thymic involution is related to an increase in the rate of the sex hormones at the time of puberty[17].

The main function of the thymus is to support maturation of LPCs into T-cells, a process called thymopoiesis. It is a complex process that starts with the colonization of the thymus primordium by LPCs, followed by their commitment to T-cell lineage, and subsequent differentiation to mature T-lymphocytes. These are characterized by the expression of T-cell receptors (TCR), associated with the co-receptor CD3, and the expression of accessory molecules such as CD4 and CD8.

In the postnatal thymus, LPCs enter at the cortico-medullary junction through blood vessels[11]. LPCs are Triple Negative (TN) cells, as they do not express the co-receptor CD3 or the accessory molecules CD4 and CD8. These TN cells can be further discriminated according to surface expression of CD44 and CD25. Sequential gain of CD25 and loss of CD44 expression, respectively, serve to delineate early differentiation steps within this subset of thymocytes[14] (Figure 2). The first maturation steps occur in the cortex where early thymocytes undergo proliferative expansion, are committed to the T-cell lineage, and start rearranging the TCR genes, the major event in thymocytes development. This process, called VDJ recombination, consists in the rearrangements in individual lymphocytes of different variable (V) region gene segments with diversity (D) and/or joining (J) gene segments. This creates a unique antigen-binding structure in each T-lymphocyte clone, leading to a highly diverse T-repertoire. In association with the TCR expression, thymocytes express the co-receptor CD3. In this phase, LPCs mature from TN to Double Positive (DP) cells, expressing CD4 and CD8. Then the DP cells selected to proceed maturation migrate into the medulla, to become $CD4^+$ or $CD8^+$ Single Positive (SP) T-lymphocytes. These cells exit the thymus through blood vessels into the peripheral immune system (Figure 2).

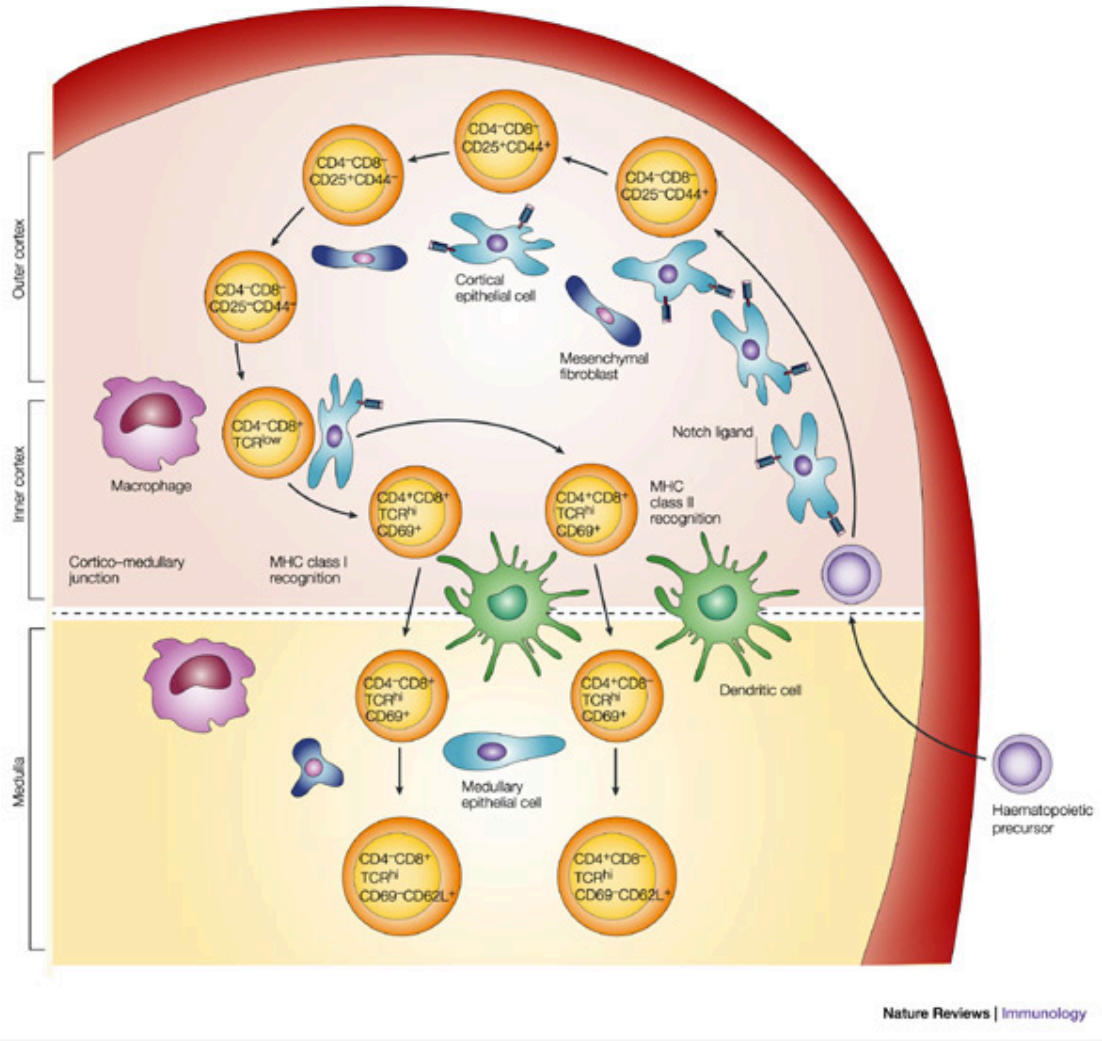

Figure 2. Thymocytes maturation pathway. The thymic architecture is organized into discrete cortical and medullary areas, each of which is characterized by the presence of particular stromal cell types, as well as thymocyte precursors at defined maturation stages. Thymocyte differentiation is characterized by the expression of well-defined cell-surface markers, including CD4, CD8, CD44 (or CD117) and CD25, as well as the status of the TCR. Interactions between Notch receptor-expressing thymocytes and thymic stromal cells that express Notch ligands induce a complex programme of T-cell maturation in the thymus, which ultimately results in the generation of self-tolerant $CD4^+$ helper T-cells and $CD8^+$ cytotoxic T-cells, which emigrate from the thymus to establish the peripheral T-cell pool. (Adapted from Zúñiga-Pflücker 2004[18])

During their maturation, thymocytes are submitted to high-stringency selection processes, where potentially harmful cells that avidly recognize self-structures may be eliminated or induced to alter their antigen receptors. In the DP stage of thymocytes maturation, Positive Selection (PS), also called death by neglect, ensures that mature T-cells are self-MHC (Major Histocompatibility Complex) restricted. In this process, thymocytes whose TCR binds to self-MHC molecules are rescued from programmed cell death, whereas thymocytes whose receptors do not recognize self-MHC die by default.

DP thymocytes in the medulla are also subjected to Negative Selection (NS) by which developing thymocytes that express self-reactive antigen receptors are eliminated, thereby contributing to the maintenance of self-tolerance. NS involves high-avidity binding of a

thymocyte to self-MHC molecules with bound peptides in thymic Antigen Presenting Cells (APCs) leading to apoptotic cell death (a phenomenon known as clonal deletion). This is an important mechanism for maintaining tolerance to many self-antigens: it is called central tolerance[16].

These selection processes, drive maturation of TCR-expressing thymocytes and shape the TCR repertoire toward self-MHC restriction and self-tolerance.

In the periphery, T-cells become activated when the TCR recognizes the complex of foreign antigens bound to a MHC molecule on the surface of APCs. $CD4^+$ T-cells, called T-helper, are class-II MHC restricted and are important in cell-mediated immunity (macrophage activation) and humoural immunity (B-cell differentiation). $CD8^+$ T-cells are the cytotoxic T-cells, class-I MHC restricted, whose function is to kill cells infected with microbes, and tumor cells[16].

The major molecules (TCR, CD3, CD4 and CD8 co-receptors) and major events in the maturation pathway of LPCs into T-cells appear to be conserved between birds and mammals, although some differences have been described[19,20].

II. The Thymus and Parathyroid Glands Development

Thymus development is intimately linked to that of the parathyroid glands as they share a common embryonic origin. These pharyngeal organs derive from the pharyngeal pouches (PPs), bilateral transient structures arising from the endoderm of the most anterior region of the foregut: the pharynx[1]. The PPs, along with the opposing pharyngeal clefts (invaginations of surface ectoderm) form the separation between pharyngeal arches, the bilateral bulges that involve the pharyngeal region[1,2] (Figure 3).

The endodermic origin of TECs was demonstrated for the first time, using the quail-chick chimeric model by Le Douarin and Jotereau[3]. In chicken and in human, thymic and parathyroid organ rudiments derive from the third and fourth PPs (3/4 PPs)[3,5] (In mouse these glands derive from the 3rd PP).

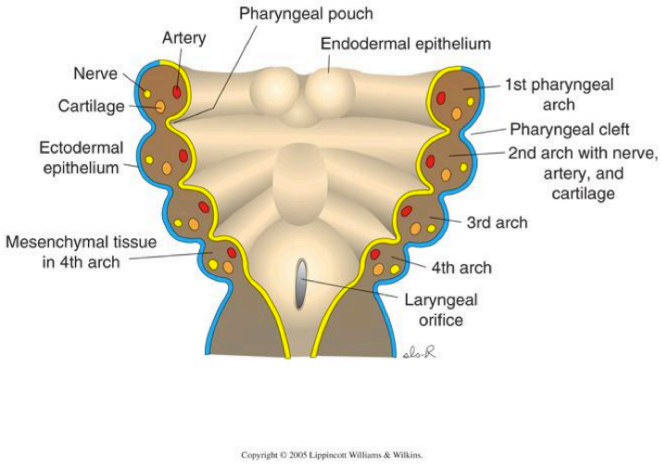

Figure 3. **Scheme representing coronal section through the pharyngeal zone**. Pharyngeal arches, consist of mesenchymal and mesodermal cells bounded by an outer layer of surface ectoderm (blue) and inner layer of pharyngeal endoderm (yellow). The ectoderm forms invaginations, the pharyngeal clefts, which separate the arches, whereas the endoderm forms the opposing outpocketings, the pharyngeal pouches.

The common primordium is patterned into organ-specific domains that can be identified by the regionalized expression of molecular markers early in organogenesis, before morphological distinctions are present[4].

In mouse, the parathyroid domain is defined by the expression of the transcription factor *glial cells missing-2 (Gcm2)* as early as embryonic day 9.5 (E9.5), before the formation of the organ primordium[4]. Thymic Epithelium (TE), is identified in a ventral position in the pouch, by the expression of the transcription factor *forkhead box N1* (*Foxn1*), detected from E11.25, coinciding with the budding off and outgrowth of the common primordium[4]. Once the primordium forms, *Gcm2* and *Foxn1* are expressed in a complementary fashion in the parathyroid- and thymus-specific regions before the division into morphologically distinct organs[4].

Foxn1 loss-of-function mutation leads to congenital athymia and hairlessness in *nude* mouse. In this mouse, LPCs fail to enter the thymic primordium and instead remain in the surrounding perithymic mesenchyme. In addition, the epithelial cells within the primordium fail to expand after E12.5 and vascularization of the primordium does not occur[12]. Thus, Foxn1 is not required for initiation of thymus organogenesis[6], but is required cell-autonomously for TECs differentiation[21].

Gcm2 may be the determining factor for parathyroid development, since, when deleted, no parathyroid glands are formed, (without interfering with thymus development)[22]. In *Gcm2*$^{-/-}$ mutant mouse, parathyroid hormone (PTH) expression is undetectable at E11.5, indicating that Gcm2 plays a crucial role very early in parathyroid organogenesis[4]. *Gcm2* is exclusively expressed in the parathyroid and its embryonic rudiment, in mammals and avians[22], and is an excellent marker of the early parathyroid domain[4,15].

In quail, *Gcm2* and *Foxn1* expression are detected at E2.5 and E3.5 respectively by RT-PCR, indicating that endoderm specification into Parathyroid Epithelium (PTE) occurs earlier than into TE[5]. In chicken, *Foxn1* and *Gcm2* transcripts occupy mutually exclusive and adjacent domains of 3/4 PPs at E5, with *Foxn1* expression located in the dorsal/anterior domain and *Gcm2* in a more ventral one[5].

Although these data are similar to those reported in the mouse, the domain of *Foxn1* expression is inverted along the dorsal-ventral axis of the pouches in chicken and quail, when compared to mouse embryos. These distinct positions during embryogenesis might contribute to the different anatomical locations of the adult thymus between mammals and birds[5].

Thymus organogenesis can be divided in two main temporal phases; an early thymocyte-independent phase, where cellular interactions between the endoderm and the surrounding mesenchyme direct TECs specification[2], followed by a thymocyte-dependent phase. At this later stage, the thymic rudiment depends on colonization by LPCs and on lympho-epithelial (LPC-TEC) interactions for further maturation of thymic epithelium into cortical and medullar compartments[6–9].

II.1 Thymus Development: thymocyte-independent phase

Using the quail-chick chimeric model, Le Douarin and Jotereau showed that the 3/4 PPs endoderm isolated from early quail embryos (E1.5 - E2.5) was able to develop into thymic epithelium (TE) with the cooperation of a heterologous mesenchyme such as the somatopleure or splanchnopleure of 3 days-old chicken embryos, which thus could be considered “permissive” to endoderm development[23]. Furthermore, the grafted endoderm was capable of inducing the heterologous mesenchyme to participate in the formation of a fully developed thymus[3,23,24]. In contrast, mesenchymal environments of the somite and limb bud were non-permissive to 3/4 PPs endoderm development[23,24]. These data provided the first evidence that the development of a functionally competent thymus depends initially on a series of epithelial-mesenchymal interactions, that direct TECs specification[2]. Moreover, they revealed that some heterologous mesenchymal tissues are able to mimic the role played by NC-derived mesenchyme during normal development of the thymus in the pharyngeal region. NC-cells are a transient population formed between the neural tube and surface ectoderm, which colonizes the branchial arches, surrounds each primordium, and later forms the perivascular mesenchyme and the capsule of the thymus[3].

II.2 Thymus Development: thymocyte-dependent phase

As pointed out above, in a second phase, thymic development depends on colonization by LPCs and on lympho-epithelial (LPC-TEC) interactions, essential for differentiation of TECs

in two different lineages, cTECs and mTECs, and for T-cell maturation[6–8]. In mouse, it was shown that this colonization step and subsequent differentiation of cTECs and mTECs are also dependent on Foxn1 activity[6].

Le Douarin first described three different waves of colonization of LPCs in avian embryogenesis[10] while in mouse only two waves are described[25]. In mouse, the first wave of colonization occurs before thymus vascularization, at E12[11], and LPCs enter the rudiment by migration trough the surrounding perithymic mesenchyme. After vascularization, LPCs enter the thymus through blood vessels (in the second wave of colonization and in adult)[26]. As the two embryonic waves behave differently, one may wonder if different molecular interactions between LPCs and TE may occur during the distinct colonization events[12]. In chicken, the first wave of colonization occurs at E6 - E6.5[3] and the pathway by which LPCs enter the thymus has not been described yet.

It was suggested that chemokines secreted by the TE and PTE guided LPCs homing into the fetal thymus. Chemokine/chemokine-receptor pairs can be categorized in two families. “Inflammatory” chemokines regulate migration of leukocytes in inflammatory conditions, and “homeostatic” chemokines, constitutively expressed, regulate migration of leukocytes and their precursors under steady-state conditions[27].

Chemokine/chemokine-receptor interactions, such as Cxcl12/Cxcr4 and Ccl25/Ccr9 pairs, have been implicated in the migration of thymocytes within the thymus[11]. In mouse, Liu et al showed that the coordination between the chemokines Ccl21 (a Ccr7 ligand) and Cccl25 (a Ccr9 ligand) is essential for guiding LPCs colonization before thymus vascularization, but not after[26]. They showed that *Ccl25* is expressed both by the Foxn1-dependent domain and the Gcm2-dependent domain of the 3rd PP, whereas *Ccl21* is expressed in the 3rd PP region exclusively by the Gcm2-dependent domain[26].

In Foxn1$^{-/-}$ mouse, there is a loss of expression of the chemokines *Ccl25* and *Cxcl12* (expressed by the fetal thymus and a ligand of Cxcr4), possibly explaining the observed lack of LPCs colonization of the thymic rudiment[2,12,28]. Moreover, when *Ccl25* expression is induced in *Foxn1*$^{-/-}$ thymic rudiment an increase in the lymphocytes number is observed in the thymic niche, compared to *nude* thymic epithelium[29].

Candidate molecules that could mediate the crosstalk between thymocytes and the thymic stroma are molecules reciprocally expressed by TECs and thymocytes, such as adhesion molecules and cytokines and their receptors. A potential example is Notch signaling pathway, since Notch-1 is expressed on early thymocytes, and Notch ligands are expressed in TECs.

III. Notch Signaling Pathway

Notch signaling is a major signaling-pathway, highly conserved between the animal kingdom, which regulates many biological processes in embryonic development, as well as differentiation and tissue homeostasis in many adult organ systems. In the last years, it became evident that Notch plays an essential role in the development of embryonic hematopoietic stem cells, and influences multiple lineage decisions of developing lymphoid and myeloid cells[30]. Notch receptors and ligands have been implicated in multiple checkpoints in thymocyte development and peripheral T-cell differentiation.

Mammals possess four Notch receptors, activated by two families of membrane-bound ligands named Jagged-1 and -2, and Delta1, 3, and 4[31]. In chicken, there are two Notch receptors, activated by Serrate-1 and -2 (Jagged homologues), and Delta ligands (Delta1, -3 and -4).

Notch activation is initiated when Notch receptors interact with ligands on neighboring cells, which results in the proteolytic release of the Notch intracellular cytoplasmic (ICN) domain of Notch receptors, catalyzed by the γ-secretase complex. The Notch intracellular domain translocates to the nucleus and binds to the transcription factor CSL. Once bound to CSL, ICN recruits other co-activators, including mastermind proteins, to form a transcriptional activation complex that induces the expression of downstream target genes. This Notch activation complex is short-lived, as nuclear ICN is targeted for degradation[31,32]. The effects of Notch signaling are dependent on dose, timing and context, and regulate cell-fate decisions (inhibit, delay or induce differentiation), cell numbers and positions through effects on proliferation and survival[31,32].

III.1 Notch signaling in thymopoiesis

Notch signaling is implicated in lymphopoiesis, particularly in thymocytes development. Specifically, Notch1 inactivation causes a complete block in T-lineage development, which suggests that Notch1 is the key Notch receptor involved in T-lineage commitment and thymic T-cell maturation *in vivo*[32–34]. The ligands responsible for Notch activation and T-cell commitment have already been identified. Jaleco et al, showed that Delta1, and not Jagged-1, completely blocks the differentiation of lymphoid progenitor cells into the B-cell lineage, while promoting the emergence of a population of cells with characteristics of a T/NK-cell precursor[13]. Moreover, *in vivo* over-expression of *Delta4* results in extra-thymic T-cell development[32], and *in vivo* inactivation of *Delta4* in mouse TECs leads to a complete block of thymopoiesis, and ectopic appearance of immature B-cells in the thymus[34,35]. Furthermore, signaling through activated Notch has been implicated in the decision processes underlying the functional bifurcation of CD4/CD8 T-lymphocytes, the choice between αβ and γδ TCR

and in maturation processes of $CD4^+$ and $CD8^+$ cells[13,36,37].

In the mouse model, Notch-related genes are differentially expressed in the adult thymic microenvironments, stressing the importance of this signaling pathway in thymic function[38,39]. TE microenvironment expresses all Notch ligands (with the exception of *Delta3,* which is hardly found in this organ[34]). Moreover, it has been shown that *Delta4* transcripts are highly expressed in the subcapsule and outer cortex regions of the thymus[14] while, *Delta1* is most abundant at the cortico-medullary junction, the point of entry of LPCs[14]. The importance of Delta1 in thymic microenvironment was highlighted when Masuda and collaborators showed that Delta1-mediated Notch signaling activation induced the appearance of a normal thymic architecture in murine fetal thymic organ cultures[40]. *Jagged-1* expression is relatively uniform in the adult thymus, showing its highest level in the central medulla, whereas *Jagged-2* displays two peaks of expression within the inner cortex and outer medulla regions[14].

As described above, when *Ccl25* expression is induced in *nude* mice thymic rudiment there is an increase in lymphocyte homing. Interestingly, when combined with induction of *Delta4* expression, thymopoiesis is restored[29].

Altogether these data suggest that both Delta1 and Delta4 may be the main Notch ligands involved in thymopoiesis.

During chicken development, preliminary data from our group showed that Notch signaling-related genes (receptors, ligands and target genes) are expressed in the prospective thymic domain and surrounding mesenchyme suggesting a role of Notch signaling at early thymic development.

IV. Objectives

In this work we studied chicken thymus (and parathyroid glands) organogenesis. Specifically, we aimed to:

1. Identify the molecular cues, namely Notch signaling related molecules, involved in thymus colonization by LPCs. For that we used chimeric embryos having quail 3/4 PP endoderm grafted into either permissive or non-permissive mesenchymal territories of chicken embryos.
2. Characterize the *in situ* expression of the transcription factors, *Foxn1* and *Gcm2* during early development of thymic and parathyroid glands rudiments (from E5 to E13).
3. Unravel the ontogenic T-cell development during the dynamic waves of thymic colonization using flow cytometry analysis of CD markers (from E11 to E18).

Materials and Methods

Avian Embryos

Fertilized chicken (*Gallus gallus*) eggs obtained from Sociedade Agrícola Quinta da Freiria, S.A., Portugal, and quail (*Coturnix coturniz japonica*) eggs obtained from Interaves**,** Sociedade Agro-Pecuária, S.A. Portugal, were stored at 16ºC and incubated at 38ºC in a humidified incubator to initiate development. Embryos age was estimated by the duration of incubation.

Riboprobes synthesis

Plasmid DNA MIDI-Preparation – Single colonies of transformed bacteria with TOPO II PCR plasmids cloned with the gene of interest were collected and inoculated into a pre-culture of 5mL of liquid LB medium supplemented with ampicillin (100µg/mL) grown at 37ºC with vigorous shaking (225rpm) up to mid-log exponential growth phase. 1mL of the pre-culture was then inoculated into 50mL of liquid LB supplemented with ampicillin (100µg/mL) and incubated overnight at 37ºC with vigorous shaking (225rpm). To purify the plasmids we used the QIAfilter Plasmid Midi kit (QIAGEN) according to the protocol recommended by the manufacturer. DNA samples were stored at -20ºC.

Riboprobes synthesis – Recombinant TOPO II PCR plasmids containing the sequence of the genes of interest were digested with the appropriate restriction enzyme in order to be linearized (Table 1): 20µg of DNA, 15µL of 10X enzyme buffer, 5µL of restriction enzyme (6U-20U/µL) and RNase-free water up to 150µL. Purification of the linearized plasmid was performed using phenol:chloroform extraction and ethanol precipitation (see appendix II). Anti-sense and sense RNA labeled probes were synthesized by "run-off" *in vitro* transcription: 8µL of Transcription Optimized 5X Buffer (Promega), 4µL of 0.1M DTT (Promega), 2µL of each rGTP, rATP, rCTP (10mM) (Roche), 1,3µL of rUTP (10mM) (Roche), 0,7µL of Digoxigenin-11-UTP (10mM) (Roche), 2µL of RNasin® Ribonuclease Inhibitor (Promega) 14µL of RNAse free water, 2µL of the appropriate RNA polymerase (see Table 1), and 2µL (2µg) of the linearized DNA template. This reaction solution was then incubated for 2h at 37ºC, and afterwards treated with 6µL of DNase I recombinant RNase-free (10U/µL) (Roche) at 37°C for 15min. The digoxigenin (DIG) labeled probe was purified using Illustra MicroSpin G-50 Columns (GE Healthcare) according to manufacturer instructions. The samples were stored at -20ºC.

Table 1. Restriction enzymes and RNA Polymerases used to linearize and synthesize the riboprobes.

Gene	Specie	Restriction Enzime	RNA Polymerase	Plasmid Source
Foxn1	Chicken	NotI	SP6	Neves et al, 2012[5]
Gcm2	Chicken	NotI	SP6	Neves et al, 2012[5]
Delta1	Chicken	EcoR1	T3	D. Henrique
Delta4	Chicken	Xho1	T7	D. Henrique

Agarose gel electrophoresis – Restriction reactions and riboprobes integrity were confirmed by agarose gel electrophoresis. UltraPureTM Agarose (Invitrogen) was dissolved by heating in 1X TAE buffer (appendix I) to a final concentration of 0.8-1.5%, depending on the required resolution for the DNA fragment or RNA.

In Situ Hybridization, Immunohistochemistry and Hematoxylin-Eosin staining

Paraffin sections – At specific developmental stages the embryos were dissected, and after removing the extra-embryonic, were fixed overnight with 3.7% paraformaldehyde at 4ºC, processed and included in paraffin. Sections were obtained in a Manual Rotary Microtome (Leica RM2235), in series of three slides with four 7µm sections each, thus allowing the comparison of different stainings in regions in close proximity in the embryo.

In Situ *Hybridization (ISH) in paraffin sections* – ISH with different riboprobes (Table 1) were performed following the protocol previously described in Neves et al. 2012[5]. Pictures were taken using an Upright Brightfield Microscope Leica DM2500, equipped with a color camera for brightfield and differential interference contrast imaging.

Immunohistochemistry (IHC) in paraffin sections – Paraffin sections were obtained as described above. Protein expression detection in paraffin sections was performed using the protocol described by Neves et al. 2012[5], and optimized for each antibody. The antibody against HNK1 (Human Natural Killer 1) antigen was used to detect cells of the peripheral nervous system[41]. HNK1 is expressed in migrating neural crest cells and in other cell types. It is a neuronally expressed adhesion molecule with a carbohydrate epitope that contains a sulfoglucuronyl residue. The QCPN (Quail non-Chick PeriNuclear antibody) antibody used targets a nuclear antigenic determinant specific to quail cells[5]. Pictures were taken using an Upright Brightfield microscope Leica DM2500, equipped with a color camera for brightfield and differential interference contrast imaging.

Hematoxylin-Eosin (HE) staining – HE staining in paraffin sections was done using Harris Hematoxylin (Merck Millipore) and Eosin Y alcoholic (Thermo scientific) according to manufacturers instructions. Pictures were taken using an Upright Brightfield microscope Leica DM2500, equipped with a color camera for brightfield and differential interference contrast imaging.

Flow Cytometry

Cells preparation - Chicken embryos were dissected at specific time points: E11, E12, E13, E15, E17 and E18. The thymi were isolated and maintained overnight at 4ºC in PBS (1X)/ 2% FBS/ Penstrep (1X). Thymocytes suspensions, pooled from at least five embryos for each developmental stage, were prepared by mechanically disrupting the thymus by gently pressing the tissue through a fine nylon mesh (pore size 70µm) to obtain a single cell suspension. The suspension was then purified by Ficoll density gradient separation (Ficoll-Paque PLUS GE Healthcare Life Sciences), to remove dead cells and red blood cells. Cell counts were performed using a haemocytometer and we used 10^5 cells for each staining (see Appendix II). Each time point analysis was repeated independently twice.

Cells staining - Cells were stained following the protocol described in Appendix II. Cells were stained using a panel of mouse anti-chicken monoclonal antibodies from Southern Biotech: anti-CD4 FITC (clone CT-4), anti-CD8α FITC (clone CT-8), anti-CD8α PE (clone CT-8) and anti-CD3 PE (clone CT-3). Antibodies concentrations used were as suggested by the manufacturer. Single stainings for all antibodies were analyzed at each time point and the results were consistent with the results obtained with combinations of antibodies (Data not shown). The combinations of antibodies used are described in Table 2.

Table 2. Flow cytometry staining combinations

	Double Stainings			
FITC	CD4	CD4	CD8	CD4/CD8
PE	CD8	CD3	CD3	CD3

Analysis - Data was acquired in a BD LSRFortessa cell analyzer. Different settings were needed to analyze stainings with CD3-PE or CD8-PE, and for thymocytes populations from older embryos (E17 and E18). For each staining condition a minimum of 10000 events was acquired, and data was analyzed with FlowJo (Tree Star, Inc. Ashland, OR). A cell gate was used to exclude debris in the events acquired, and then with a singlet gate we defined the population of thymocytes to study. Afterwards, cut-offs between negative and positive populations were defined individually. The proportions of CD3, CD4 and CD8 defined cell

populations are expressed as a percentages of thymocytes, and the mean and standard deviation between the two independent experiments for each condition were calculated.

Quail-Chick Chimeras

Quail embryos were dissected at E3, and the pharyngeal region isolated and treated with a solution of pancreatin (8mg/ml, Sigma) for 60 min on ice, allowing full separation of the endoderm of the 3/4 PP from the mesenchyme. The isolated endoderm was grafted in chicken embryos either in the somatopleural mesenchyme, or in the limb bud mesenchyme, at E2.5 - E3 (Figure 4). The chimeric embryos were incubated at 38ºC in a humidified incubator for further development. Embryos were sacrificed 3 or 5 days post-grafting and processed according to the method used afterwards.

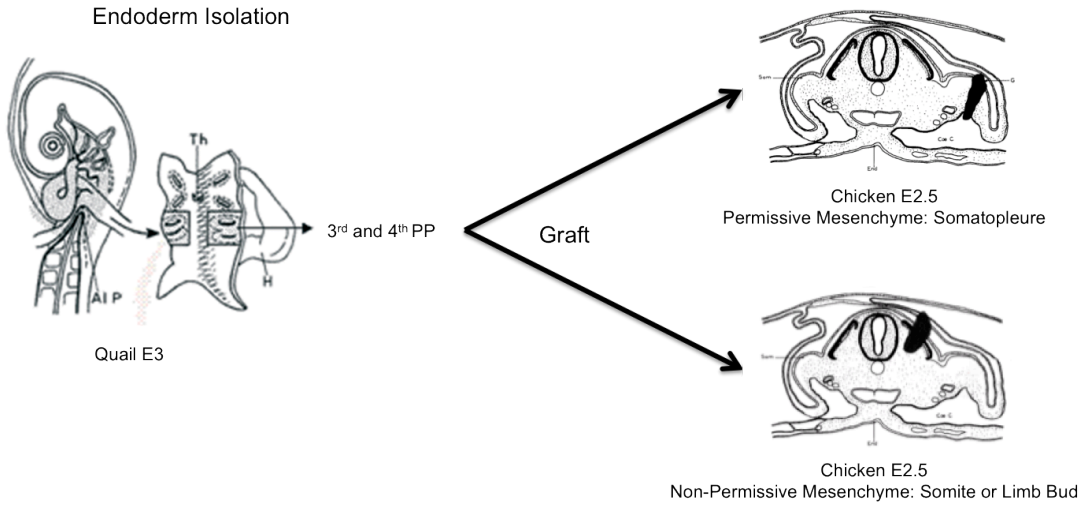

Figure 4. Quail-chick chimeric model:.Quail endoderm (E3) is grafted in the mesenchymal territories of the somatopleure or of the limb bud of chicken embryos (E2.5 - E3).

Results

I. Notch signaling in thymus epithelium colonization by LPCs

In 1967, Le Douarin using the quail-chick model showed that distinct mesenchymal tissues are permissive (somatopleural mesenchyme) or non-permissive (limb bud mesenchyme) to sustain 3/4 PPs endoderm development in a functional thymus[23].
Recently, our group showed that 3/4 PPs endoderm specified into TE when grown in permissive or in non-permissive mesenchymal territories. However, TE colonization by LPCs occurred only when endoderm is developed in permissive environments (somatopleural mesenchyme)[5].

Here, our aim was to investigate the ligands of Notch signaling involved in thymic epithelium colonization. For that we developed quail-chick chimeras as previously described. Briefly, E3 quail 3/4 PPs endoderm was isolated and grafted into distinct mesenchymal territories, either in the somatopleural or the limb bud territory, of E2.5 chicken embryos. Due to the complex manipulation of grafting procedures, a slight delay in the endoderm development was observed in chimeric embryos. The chimeric embryos were allowed to develop for 3 or 5 days, stages corresponding to E5 and E7 of the grafted endoderm. The first wave of colonization by LPCs occurs in the middle of this time-window (at E6 in quail). We analyzed the expression of Notch ligands, *Delta1* and *Delta4*, known to be expressed in the thymus and to support T-cell differentiation[13,38,42]. The grafted endoderm was identified by screening paraffin sections of chimeric embryos stained by immunohistochemistry with QCPN (specific for quail cells) (Figures 5A-D). Sequential section slides were then used to analyze the expression of *Foxn1*, *Delta1* and *Delta4*, by *in situ* hybridization. The neural tube expression of *Delta1* and *Delta4* of the chimeric embryos was used as an internal control. Controls for *Foxn1* expression were performed in parallel using sections with thymi of E13 chicken embryos (data not shown).
In 3- and 5-days chimeric embryos, we observed *Delta1* expression in the endoderm grafted in the permissive mesenchyme (n=1 for each condition; Figures 5E, 5G). In contrast, when grafted into the non-permissive limb bud mesenchymal territory, low or no expression of *Delta1* was observed in the developing endoderm (n=1 for each condition; Figures 5F, 5H). Moreover, *Delta1* expression was up-regulated in the limb bud mesenchyme in the vicinity of the grafted endoderm in chimeric embryos with 5 days of development (n=1; Figure 5H), but not in the somatopleural mesenchyme (Figures 5E, 5G).

The other Notch-ligand studied, *Delta4*, was strongly expressed in the endoderm grafted in either mesenchymal environments, both after 3 and 5 days of chimeric embryos development (n=1 for each condition; Figures 5I-L). No *Delta4* expression was observed in the surrounding mesenchyme in any condition (Figures 5I-L).

We confirmed that *Foxn1* is faintly expressed in the endoderm of the chimeric embryos analyzed, in both permissive and non-permissive environments (n=1 for each condition; data not shown).

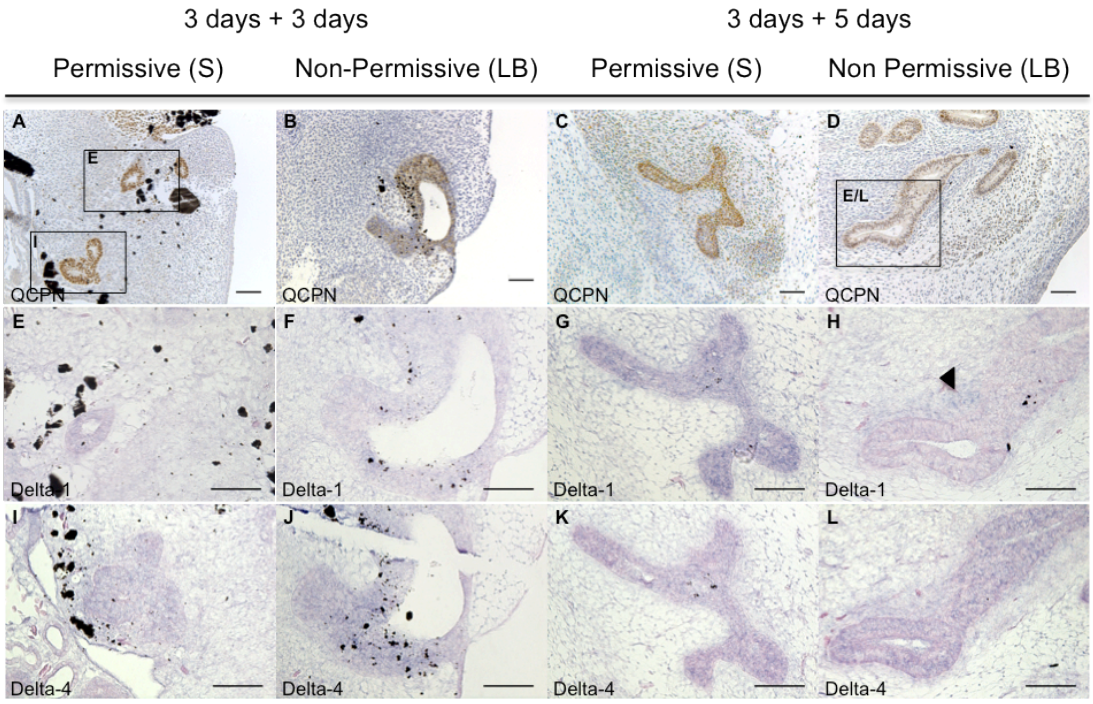

Figure 5. *Delta-1* and *Delta-4* expression in the grafted quail endoderm and surrounding chick mesenchyme of chimeric embryos. Transverse sections of chicken host embryos after grafting quail E3 endoderm in the Somatopleural (A,E,I; C,G,K) or in the Limb Bud (B,F,J; D,H,L) mesenchyme of chicken E2.5. Chimeric embryos developed for 3 days (A,E,I; B,F,J) or 5 days (C,G,K; D,H,L). Sequential paraffin sections were stained with QCPN antibody (A-D), or processed for *in situ* hybridization with *Delta1* probe (E-H) and *Delta4* probe (I-L). (S: Somatopleural mesenchyme; LB: Limb Bud mesenchyme). Scale bars 20µm.

Together our data showed that *Delta1*, and not *Delta4*, is specifically down-regulated in the endoderm developed in the non-permissive mesenchymal territory (limb bud), where no LPCs colonization is observed, and up-regulated in the adjacent mesenchyme.

II. Formation of chicken pharyngeal organs: thymus and parathyroid glands

In chicken, the thymus and parathyroid glands share a common embryonic origin, the endoderm of the 3/4 PPs. Their development is initiated with the budding-off of the pouches, followed by their patterning into organ-specific domains. These domains can be identified by the expression of the specific transcription factors *Foxn1* and *Gcm2,* for the thymus and parathyroid, respectively[5]. Afterwards, the rudiments detach from the pharynx and migrate to their final locations.

We studied the development of these glands in chicken embryos from E5 onwards, by *in situ* hybridization technique. We analysed the expression of *Foxn1* and *Gcm2*, in sequential slide sections of the neck region of chicken embryos.

First, we identified the area of interest (the glands rudiments) by Hematoxylin-Eosin (HE) staining in slide sections of E5 embryos (Figure 6A). The adjacent slides were then used to detect *Foxn1* and *Gcm2* expression. We observed that these genes were faintly expressed in the pouches (Figures 6B, 6C).

At later stages, organs primordia were identified by morphological analysis combined with HNK1 (Human Natural Killer 1) detection by immunohistochemistry. This marker is expressed in migrating neural crest cells and derivatives and in other cell types (like NK-cells) allowing a better determination of the area of interest (the glands rudiments)[41]. From E5 onwards, *Foxn1* and *Gcm2* were detected in the epithelia of the organs primordia, of the thymus and parathyroid glands, respectively, at all developmental stages studied (Figures 6B, 6E, 6H, 6L, 6Q and 6C, 6F, 6I and 6T).

As observed in Figures 6D-F, at E7, organs primordia were detached from the pharynx, separated from each other and near the nodose ganglion, as previously described[2,5]. Two days later (E9), the parathyroid glands had already a cordonal/tubular structure, characteristic of the mature organ (Figure 6I). The thymus formed a consistent cord-like structure, expressing *Foxn1* transcripts in epithelial cells, intermixed with very few non-expressing large cells, probably corresponding to homing of hematopoietic progenitors cells (Figure 6H).

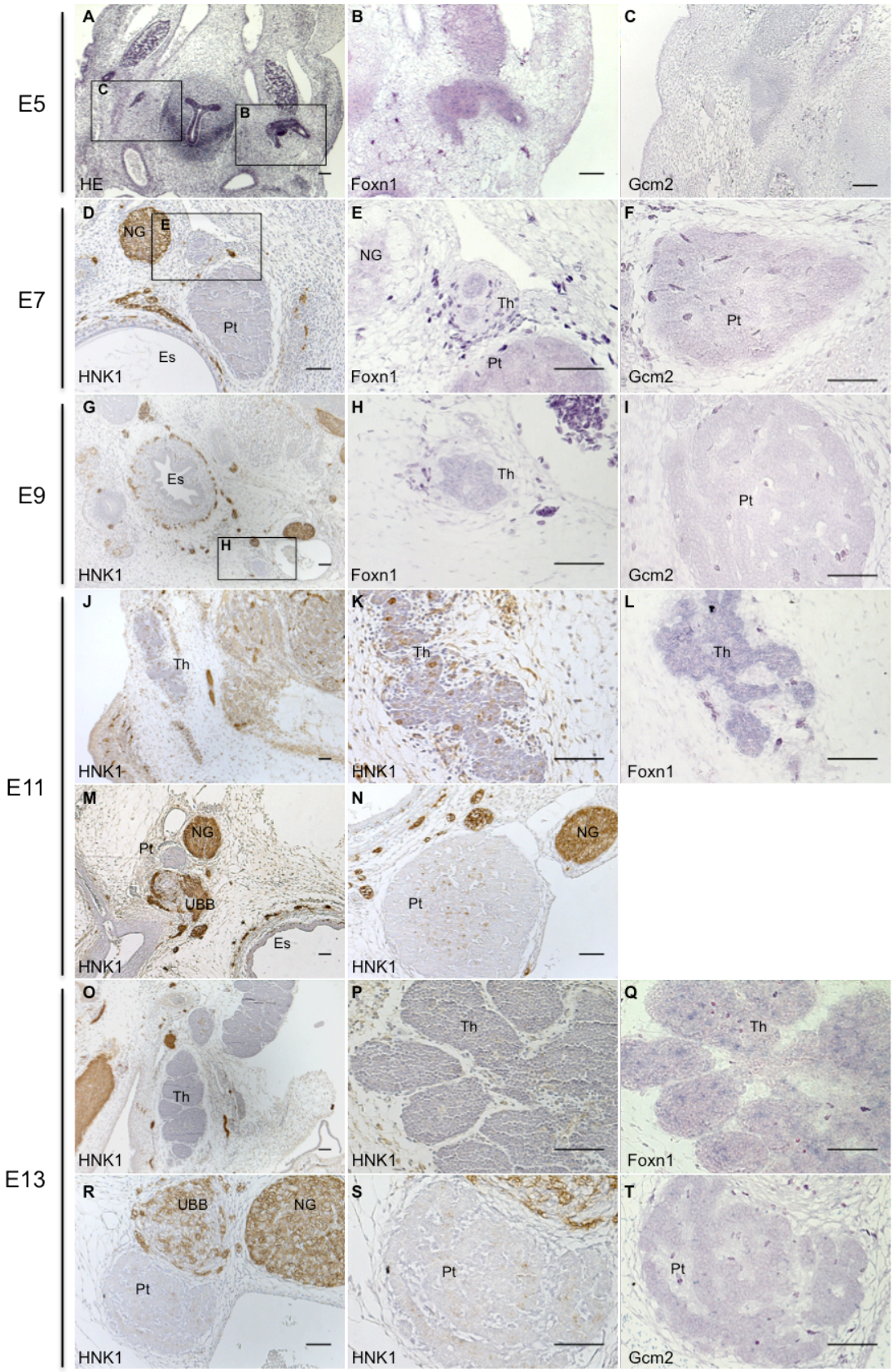

Figure 6. The expression of *Foxn1* and *Gcm2* in developing epithelia of the thymus and parathyroid glands in chicken embryos. Transverse sections of chicken embryos of E5 (A-C), E7 (D-F), E9 (G-I), E11 (J-N) and E13 (O-T), stained with HE (A), HNK1 (D,G,J, K, M, N, O, P, R and S). B, E, H, L and Q were hybridized with *Foxn1* riboprobe; and C, F, I and T with *Gcm2* riboprobe. (Es, esophagus; NG, nodose ganglion; Pt, parathyroid glands; Th, thymus; UBB, ultimobranchial body).

At E11, the thymus was a tiny cord-like structure elongated along the neck of the embryos, and already lobulated, as seen in Figures 6J and 6K. Again, between the epithelial *Foxn1* positive cells, we observed few negative cells, suggesting that there were still very few LPCs at this stage in the thymus (Figure 6L). As observed in Figures 6O-Q, the different thymic lobes at E13 were very lobulated and exhibit an increased number of *Foxn1* negative cells when compared to E11. Moreover the separation between cortex and medulla was discernible at this stage of thymic development. At these two later stages, HNK1 staining in the thymus was observed (Figure 6J, 6K, 6O, 6P), suggesting the presence of Natural-Killer (NK)-cells.

Gcm2 expression was observed at E11 (data not shown) and at E13 (Figure 6T). At these later stages, the parathyroid glands maintained their anatomical position, near the nodose ganglion and the ultimobranchial body (Figures 6M, 6R), and increased in size (data not shown).

With this study, we confirmed that at E7 the rudiments have separated from the pharynx and from each other. We observed that at E9 the thymus forms a consistent cord-like structure that starts elongating along the neck. At E11, it has already subdivided into several lobes, that two days latter had clear lobules. In contrast, the parathyroid glands, at E9, are already located at their final anatomical position exhibiting the morphology of the mature organ. Moreover, we observed *Foxn1* and *Gcm2* expression in thymic and parathyroid epithelia of the rudiments until E13, respectively.

III. Thymocytes population kinetics during chicken embryo development

During chicken embryonic development, the thymus is colonized by LPCs in cyclic waves, at E6.5, E12 and E18[10,43,44]. Once inside the thymus, the progenitors adopt T-cell lineage fate, and undergo a sequence of developmental events leading to the generation of different functional lineages[45]. These developmental stages of thymocytes can be discriminated according to surface expression of molecular markers.

To study the dynamics of thymopoiesis, during chicken thymus organogenesis, we analysed the expression of the co-receptor CD3 and accessory molecules CD4 and CD8, by flow cytometry, using different antibodies combinations (Table 2 in Materials and Methods; dot blots representing the percentages of thymocytes co-expressing CD3 and CD4 or CD8 shown in Appendix III, Figure 11). The study was performed from E11 onwards, since very few hematopoietic cells could be harvested from the thymic rudiment at earlier stages, preventing a representative cytometry analysis. The results are presented in Figure 7 and Table 3, as percentages of thymocytes expressing the molecular markers studied.

At E11, after the first wave (at E6) and before the second wave (at E12)[10] of colonization, each of the three markers studied is expressed by a small percentage of cells. The percentage of thymocytes expressing CD8 showed a sharp increase from E13 onwards, while $CD4^+$ cells presented a similar increase with a short delay (Figure 7: green and red curves, respectively). We observed that the percentage of $CD3^+$ cells rose at two different time-points, between E12 and E13 (up to 23.95%), and between E15 and E17 (up to 67.1%) (Figure 7: purple curve; Table 3).

At E18, the oldest time-point analyzed, 89.50% of the population expressed CD8, 82.95% expressed CD4 and 65.20% expressed the CD3 co-receptor (Table 3). As expected, the proportion of TN cells decreases during development, from more than 90% at E11 to less than 5% at E18 (Figure 7: blue curve).

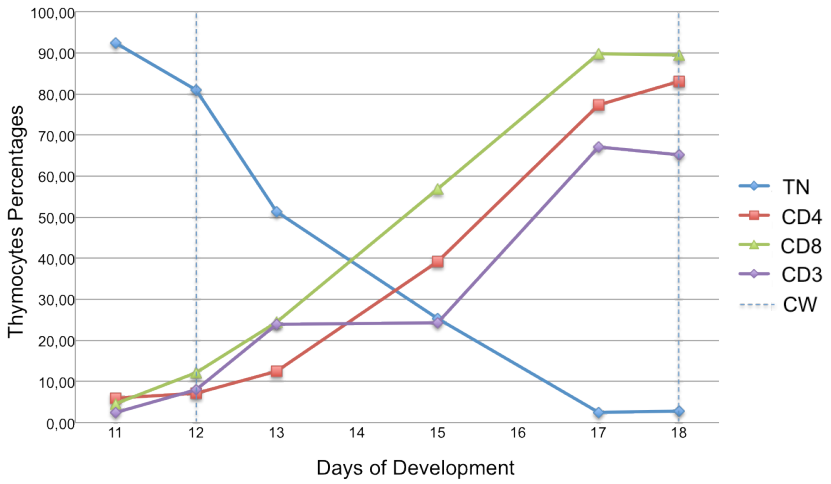

Figure 7. Percentages of thymocytes expressing CD3, CD4 and CD8 during chicken thymus ontogeny. Flow cytometry analysis of CD3, CD4 and CD8 expression in thymocytes harvested from E11, E12, E13, E15, E17 and E18 thymi. Triple Negative (TN) thymocytes ($CD3^-CD4^-CD8^-$); and single staining for CD4, CD8 and CD3. CW: Colonization Waves (timing of the 2nd and 3rd colonization waves by LPCs at E12 and E18).

Distinct populations were discriminated according to CD3 expression levels, $CD3^{low}$ and $CD3^{high}$. Until E15, all $CD3^+$ thymocytes had a low expression of CD3. In contrast, at later stages of development (E17 and E18), when CD3 expression reaches its maximum (over 60%), 10-15% of the thymocytes presented a high level of CD3 expression (Figure 8).

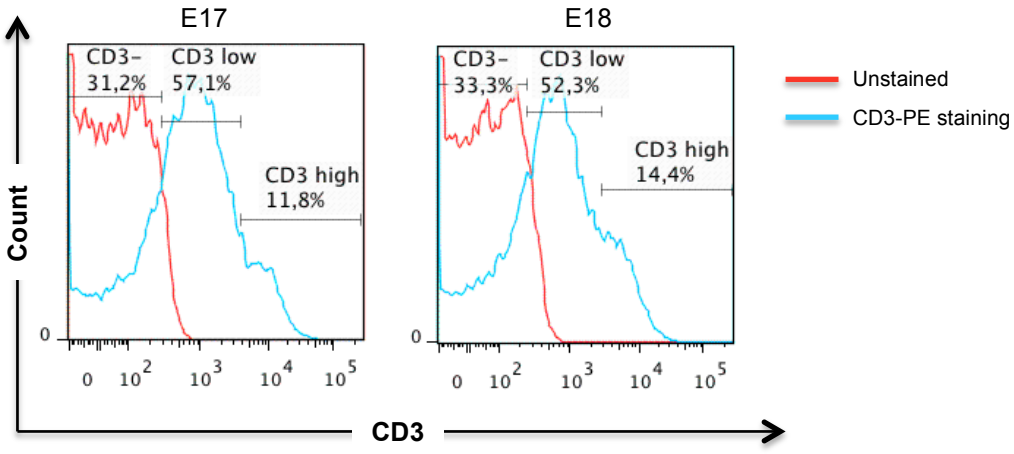

Figure 8. Percentages of thymocytes expressing different CD3 levels at E17 and E18. In red the unstained thymocytes curve, and in blue the CD3-PE staining.

Moreover, between E13 and E15, we observed an increase of DP thymocytes, $CD4^+CD8^+$, from 3.11% to 32.90%. In the case of double positive populations for $CD4^+CD3^+$ or $CD8^+CD3^+$ an increase in cells percentage was observed between E15 (less than 5%) and E17 (above 50%) (Table 3).

Table 3. Percentages in thymocytes population expressing CD3, CD4 and CD8.

	E11		E12		E13		E15		E17		E18	
	Mean	*STD*	Mean	*STD*	Mean	*STD*	Mean	*STD*	Mean	*STD*	Mean	*STD*
$CD4^+$	**5,97**	*1,97*	**7,08**	*0,16*	**12,55**	*2,19*	**39,15**	*2,90*	**77,35**	*2,76*	**82,95**	*1,06*
$CD8^+$	**4,62**	*1,36*	**12,13**	*3,08*	**24,50**	*4,10*	**56,85**	*6,29*	**89,85**	*1,06*	**89,50**	*0,57*
$CD3^+$	**2,45**	*2,41*	**8,03**	*0,94*	**23,95**	*3,18*	**24,35**	*3,75*	**67,10**	*6,08*	**65,20**	*3,68*
$CD3^+CD4^+$	**0,81**	*0,95*	**0,29**	*0,05*	**0,89**	*0,13*	**1,43**	*0,42*	**55,50**	*3,68*	**52,90**	*14,42*
$CD3^+CD8+$	**0,22**	*0,25*	**0,85**	*0,14*	**2,40**	*0,13*	**3,62**	*1,66*	**58,05**	*6,15*	**57,15**	*3,18*
$CD4^+CD8^+$	**0,22**	*0,12*	**0,59**	*0,14*	**3,11**	*1,61*	**32,90**	*3,54*	**44,20**	*1,56*	**77,60**	*1,56*
$CD3^+CD4^-CD8^-$	**7,04**		**12,25**	*1,77*	**21,45**	*10,82*	**16,80**		**6,72**		**7,39**	*1,41*
$CD3^-CD4^-CD8^-$	**92,40**		**80,90**	*0,00*	**51,25**	*4,45*	**25,40**		**2,48**		**2,81**	*1,04*

Percentages of thymocytes expressing CD3, CD4 and CD8 at all stages analyzed (STD: standard deviation).

In summary, before E13 we observed two immature thymocyte populations ($CD8^+CD4^-CD3^-$ and $CD3^+CD8^-CD4^+$) derived from the first wave of colonization. After E13, the percentage of $CD4^+CD8^+$ (DP) cells increases. Moreover, at E17 and E18, a small percentage of $CD3^+$ thymocytes expressed high levels of CD3 co-receptor.

Discussion

Understanding the steps that lead to the development of a functional thymus, a complex process crucial for correct thymopoiesis, is of major importance to understand the building of a healthy immune system.

In this work, the first aim was to study the molecular cues involved in TE colonization by LPCs using the quail-chick chimeric model. Specifically, we studied the involvement of distinct Notch-ligands in this process. We showed that, in conditions without TE colonization by LPCs, *Delta1,* and not *Delta4*, was down-regulated in the epithelium.

Secondly, we described histologically the development of the thymus and parathyroid glands by *in situ* expression of the transcription factors, *Foxn1* and *Gcm2,* respectively. We observed that, from E5 to E13, the expression of both transcription factors was maintained in the thymic and parathyroid rudiments.

Finally, we analysed by flow cytometry the maturation of the population of thymocytes during chicken thymus organogenesis. The analysis showed that at early stages (before E13) of thymic development, two immature populations emerged ($CD8^+CD4^-CD3^-$ and $CD3^+CD8^-CD4^-$), while after E13 a strong increase of a more mature population of DP cells ($CD4^+CD8^+$) was observed. This shift corresponds to the timing of the second colonization wave and the establishment of cortical and medullar compartments.

I. Delta-1 ligand is involved in early colonization of the thymic rudiment by LPCs

The colonization of thymic epithelium is a crucial and early event in thymus organogenesis. Recent results from our lab, using the quail-chick chimeric model, showed that 3/4 PPs endoderm when developed in a non-permissive environment was able to specify into TE ($Foxn1^+$), but not to be colonized by LPCs[5]. In this study, we used this avian chimeric model approach to identify the molecular cues involved in thymic epithelium colonization. We identified *Delta1*, as the specific Notch-ligand of the Delta-family involved in this process. *Delta1*, but not *Delta4*, was down-regulated in the endoderm grown in the non-permissive environment, whereas in the endoderm grown in the permissive environment, where LPCs colonization occurred, *Delta1* expression was maintained (Figure 9).

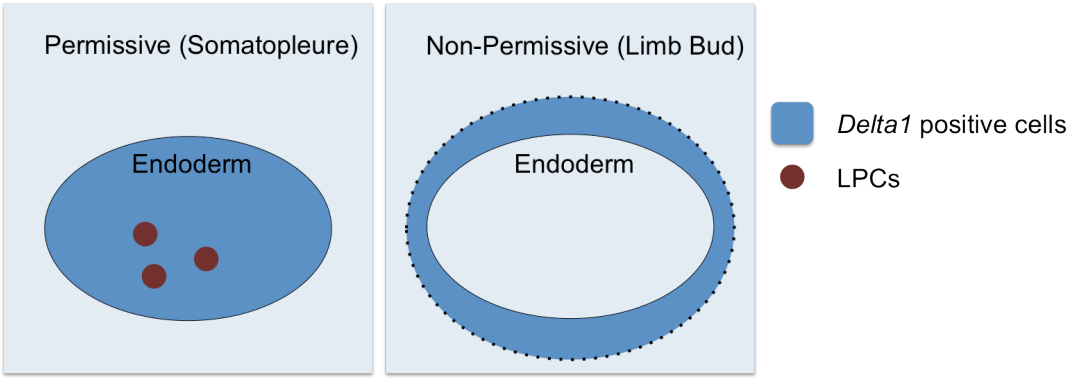

Figure 9. *Delta1* expression in the endoderm and surrounding mesenchyme in chimeras developed for 5 days. Schematic representation of *Delta1* expression in chimeric embryos developed for further 5 days.

In agreement, expression of *Delta1* was reported in the cortico-medullary junction of adult mice thymus, the entry region of LPCs. Other reports showed that *in vitro* expression of *Delta1* in stromal cells induces LPCs commitment to T-lineage[13]. Together, these data suggest that Delta1 ligand expressed by TECs might be necessary to a successful seeding of LPCs when entering the thymus.

However, we cannot exclude that other Notch ligands of the Serrate family may also be involved in this event. In fact, we observed the expression of *Serrate-1* in the 3rd PP endoderm at E3 and E4, and of *Serrate-2* in the mesenchyme surrounding the 3rd PP (3rd PA) (unpublished data), suggesting that these ligands may be involved in early stages of thymus organogenesis.

In the 5-days post-grafting chimeric embryos, we observed that the down-regulation of *Delta1* in the developing endoderm was accompanied by its up-regulation in the surrounding non-permissive mesenchyme (limb bud). This ectopic expression of *Delta1* in the neighboring mesenchyme further suggests distinct epithelial-mesenchymal interactions that might prevent thymic colonization. This interaction (between TE - Delta1^{-} with non-permissive mesenchyme - Delta1^{+}) may prevent either thymus vascularization and/or expression of chemokines involved in directing LPCs migration towards the thymic rudiment[12,26].

In the future, it will be of interest to study TE vascularization in both environments, and to analyze the expression of other molecular cues that could be involved in the process, such as Ephrin receptors, vascular endothelial growth factor families and other ligands of Notch signaling, and chemokines. Furthermore, the presence of LPCs in the surrounding mesenchyme should be assessed to help understand the possible role of chemokines and vascularization.

It is noteworthy that these results should be confirmed by the analysis of more experimental cases. The impossibility to show more cases was due to the time-consuming intensive train

to master the manipulation of isolating embryonic tissues (namely the 3/4 PPs endoderm). In addition, chimeric embryos showed low viability.
Besides, this study and the previous one[5] were performed in chimeric embryos developed for 5 days after grafting, making it impossible to determine if thymic colonization was completely abolished or merely delayed. To clarify this issue, quail-chick chimeric embryos should be developed for longer periods (until 10 days of development post-grafting).
Finally, another important study should be performed: to show Notch activation in colonizing LPCs. These experiments will reinforce the possible role of Delta-ligands (expressed in TECs) during these early events in thymic organogenesis.

II. Ontogeny of chicken thymus and parathyroid glands: histological analysis

The development of the thymus is intimately linked to that of the parathyroid glands as the epithelia of these organs share the same embryological origin: the endoderm of the 3/4 PPs (in chicken)[3]. Following initiation of organogenesis, the common rudiment is patterned into specific organ domains, defined by specific transcription factors: Foxn1, required for thymus development, and Gcm2 required for parathyroid glands development[4].
In this study, we confirmed that thymic epithelial cells maintain *Foxn1* expression until E13. Moreover, at this stage *Foxn1* expression was observed in both compartments, cortical and medullar, as in mouse[4]. We also showed that the parathyroid glands maintain *Gcm2* expression from E5 to E13. Altogether, these data suggest that Foxn1 and Gcm2 are required in the mature organs, as described in mouse[4]. Although the levels of gene expression are not comparable by *in situ* hybridization, it seems that the expression of the two transcription factors increases over time. A Real Time-PCR analysis will be needed to confirm this hypothesis.
In the future, it will be of importance to define the exact developmental stages of organs vascularization, namely of the thymus, since this event is crucial for LPCs homing.

III. Ontogeny of chicken thymus: characterization of thymocytes maturation

In chicken, the first colonization wave of LPCs enters the thymus at E6.5[3], but very few thymocytes are observed in embryonic sections until E13 (as described above). At E13, the second wave is completed[10], and both thymic compartments are distinguishable[20]. The last colonization influx of LPCs during embryogenesis occurs around E18[44].

We analyzed the dynamic of acquisition of differentiation markers during thymopoiesis. CD8 was the marker more expressed in early thymocytes (until E13) and very few cells co-expressed it with the other markers studied, CD4 or CD3 (around 20% $CD8^+CD4^-CD3^-$ thymocytes, at E13). Another single positive population was observed at the same stages of development, the $CD3^+CD4^-CD8^-$ (around 20%). These results suggest that these immature populations, $CD8^+CD3^-CD4^-$ and $CD3^+CD8^-CD4^-$, emerging after the first wave of colonization, may be important in early phases of thymus organogenesis, possibly contributing to the specification of TECs into different lineages (mTECs and cTECs), and consequently allowing the establishment of the future thymic compartments: cortex and medulla (Figure 10).

From E15 onwards, an expansion of maturing thymocytes $CD4^+CD8^+$, DP cells, in the rudiment was observed. This phenomenon occurred after the second wave of LPCs and coincided with the maturation of the thymus in cortex and medulla. These observations suggest that the second wave of LPCs has a higher T-cell progenitor activity, with thymocytes expansion and maturation into T-cells (Figure 10).

Together, our data in the avian model is in agreement with what is hypothesized in the mouse model, where evidence suggest that the first LPCs colonizing the thymic rudiment have low T-cell progenitor activity, when compared to the second wave of LPCs entering the rudiment[11].

At E17 and E18, most of $CD3^+$ cells are also $CD4^+$ and $CD8^+$ because few $CD3^+CD4^-CD8^-$ are present (less than 10%), and CD3 is co-expressed with CD4 or CD8 in more than 50% of the thymocytes population. Moreover, at these stages two levels of CD3 expression are distinguishable (low and high). These might be related with the maturation stage of thymocytes, where the more mature cells express higher levels of CD3, or with the usage of distinct TCR by the thymocytes (γδ or αβ).

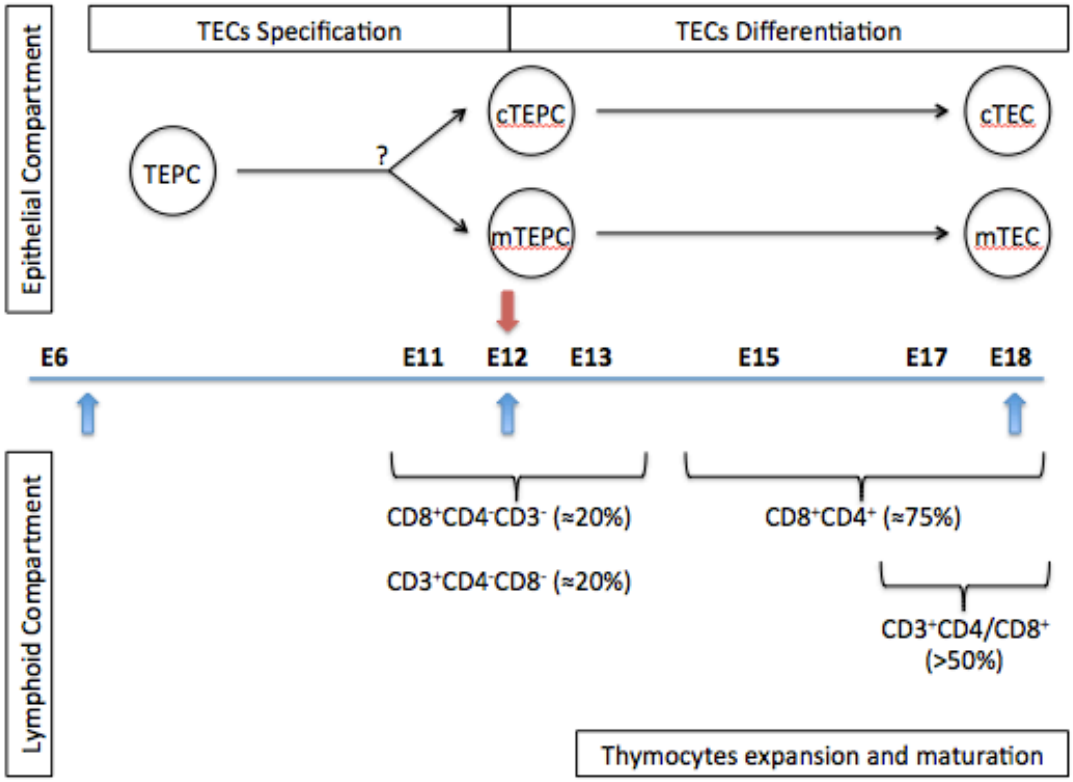

Figure 10. Correlation between thymocytes populations and thymic epithelial cells specification and differentiation during thymus development. Blue arrows: timing of LPCs colonization waves. Red arrow: Establishment of cortex and medulla. (TEPC: Thymic Epithelial Progenitors Cells).

In the future, it would be interesting to analyse the expression of the different TCR in thymocytes during ontogeny, to identify the correlation between their expression and the co-receptors expression. Another interesting study, would be to analyse the expression of these molecular markers in thymocytes at earlier stages of embryogenesis. In order to do that, another technique should be used, since very few thymocytes can be harvested from younger thymic rudiment for a consistent cytometry analysis. A possible approach is to use an immunohistochemistry analysis that would additionally identify the localization of the molecules within the thymus.

Bibliography

1. Gordon, J. & Manley, N. R. Mechanisms of thymus organogenesis and morphogenesis. *Development (Cambridge, England)* **138**, 3865–3878 (2011).

2. Rodewald, H. Thymus organogenesis. *Annual review of immunology* **26**, 355–388 (2008).

3. Douarin, N. L. & Jotereau, F. Tracing of cells of the avian thymus through embryonic life in interspecific chimeras. *The Journal of experimental medicine* **142**, 17–40 (1975).

4. Gordon, J., Bennett, a R., Blackburn, C. C. & Manley, N. R. Gcm2 and Foxn1 mark early parathyroid- and thymus-specific domains in the developing third pharyngeal pouch. *Mechanisms of development* **103**, 141–143 (2001).

5. Neves, H., Dupin, E., Parreira, L. & Le Douarin, N. M. Modulation of Bmp4 signalling in the epithelial-mesenchymal interactions that take place in early thymus and parathyroid development in avian embryos. *Developmental biology* **361**, 208–219 (2012).

6. Nehls, M; Kyewski, B; Messerle, M; Waldschütz, R; Schüddekopf, K; Smith, AJH; Boehm, T. Two genetically separable steps in the differentiation of thymic epithelium. *Science (New York, N.Y.)* **272**, 886–889 (1996).

7. Alves, N. L., Huntington, N. D., Rodewald, H.-R. & Di Santo, J. P. Thymic epithelial cells: the multi-tasking framework of the T cell “cradle”. *Trends in immunology* **30**, 468–474 (2009).

8. Anderson, G. & Jenkinson, E. J. Lymphostromal interactions in thymic development and function. *Nature reviews Immunology* **1**, 31–40 (2001).

9. Douarin, N. M. L., Dieterlen-Lièvre, F. & Oliver, P. D. Ontogeny of primary lymphoid organs and lymphoid stem cells. *The American journal of anatomy* **170**, 261–299 (1984).

10. Jotereau, F. V. & Le Douarin, N. M. Demonstration of a cyclic renewal of the lymphocyte precursor cells in the quail thymus during embryonic and perinatal life. *Journal of immunology (Baltimore, Md. : 1950)* **129**, 1869–1877 (1982).

11. Nowell, C., Farley, A. & Blackburn, C. Thymus organogenesis and development of the thymic stroma. *Methods in Molecular Biology* **380**, 125–162 (2007).

12. Itoi, M., Kawamoto, H., Katsura, Y. & Amagai, T. Two distinct steps of immigration of hematopoietic progenitors into the early thymus anlage. *International immunology* **13**, 1203–1211 (2001).

13. Jaleco, AC; Neves, H; Hooijberg, E; Gameiro, P; Clode, N; Haury, M; Henrique, D; Parreira, L. Differential effects of Notch ligands Delta-1 and Jagged-1 in human lymphoid differentiation. *The Journal of experimental medicine* **194**, 991–1002 (2001).

14. MohtashamiM; Shah, DK; Nakase, H; Kianizad, K; Petrie, HT; Zúñiga-Pflücker, J. Direct comparison of Dll1- and Dll4-mediated Notch activation levels shows differential lymphomyeloid lineage commitment outcomes. *Journal of immunology (Baltimore, Md. : 1950)* **185**, 867–876 (2010).

15. Grevellec, A., Graham, A. & Tucker, A. S. Shh signalling restricts the expression of Gcm2 and controls the position of the developing parathyroids. *Developmental biology* **353**, 194–205 (2011).

16. Abbas, Abdul K.; Lichtman, Andrew H.; Pillai, S. *Cellular and Molecular Immunology*. (2007).

17. Tarek, K., Mohamed, M., Omar, B. & Hassina, B. Morpho-histological study of the thymus of broiler chickens during post-hashing age. *International Journal of Poultry Science* **11**, 78–80 (2012).

18. Zúñiga-Pflücker, J. T-cell development made simple. *Nature Reviews Immunology* **4,** 67-72 (2004).

19. Davidson, N. J. & Boyd, R. L. Delineation of chicken thymocytes by CD3-TCR complex, CD4 and CD8 antigen expression reveals phylogenically conserved and novel thymocyte subsets. *International immunology* **4**, 1175–1182 (1992).

20. Bucy, R. P., Chen, C. H. & Cooper, M. D. Ontogeny of T cell receptors in the chicken thymus. *Journal of immunology (Baltimore, Md. : 1950)* **144**, 1161–1168 (1990).

21. Blackburn, C C; Augustine, C L; Li, R; Harvey, R P; Malin, M a; Boyd, R L; Miller, J F; Morahan, G. The nu gene acts cell-autonomously and is required for differentiation of thymic epithelial progenitors. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 5742–5746 (1996).

22. Graham, A. & Richardson, J. Developmental and evolutionary origins of the pharyngeal apparatus. *EvoDevo* **3**, 24 (2012).

23. Douarin, N. L. Influence de la nature et du degré d'évolution du mésoderme sur la différenciation de l'endoderme pharyngien chez l'embryon de poulet. *Comptes Rendus Soc. Biologie* **2**, 431–434 (1967).

24. Douarin, N. L., Bussonnet, C. & Chaumont, F. Étude des capacités de différenciation et du rôle morphogène de l'endoderme pharyngien chez l'embryon d'oiseau. *Annales D'Embryologie et de Morphogenèse* **1**, 29–39 (1968).

25. F Jotereau, F Heuze, V Salomon-Vie, H. G. Cell kinetics in the fetal mouse thymus: precursor cell input, proliferation, and emigration. *The Journal of Immunology* **138**, 1026–1030 (1987).

26. Liu, Cunlan; Saito, Fumi; Liu, Zhijie; Lei, Yu; Uehara, Shoji; Love, Paul; Lipp, Martin; Kondo, Shunzo; Manley, Nancy; Takahama, Y. Coordination between CCR7- and CCR9-mediated chemokine signals in prevascular fetal thymus colonization. *Blood* **108**, 2531–2539 (2006).

27. Heinzel, K., Benz, C. & Bleul, C. C. A silent chemokine receptor regulates steady-state leukocyte homing in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 8421–8426 (2007).

28. Liu, Cunlan; Ueno, Tomoo; Kuse, Sachiyo; Saito, Fumi; Nitta, Takeshi; Piali, Luca; Nakano, Hideki; Kakiuchi, Terutaka; Lipp, Martin; Hollander,; Georg a; Takahama, Y. The role of CCL21 in recruitment of T-precursor cells to fetal thymi. *Blood* **105**, 31–39 (2005).

29. Calderón, L. & Boehm, T. Synergistic, Context-Dependent, and Hierarchical Functions of Epithelial Components in Thymic Microenvironments. *Cell* **1**, 159–172 (2012).

30. Radtke, F., Fasnacht, N. & MacDonald, H. Notch signaling in the immune system. *Immunity* **32**, 14–27 (2010).

31. Lai, E. C. Notch signaling: control of cell communication and cell fate. *Development (Cambridge, England)* **131**, 965–973 (2004).

32. Maillard, I., Fang, T. & Pear, W. S. Regulation of lymphoid development, differentiation, and function by the Notch pathway. *Annual review of immunology* **23**, 945–974 (2005).

33. Radtke, F. *et al.* Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* **10**, 547–558 (1999).

34. Koch, Ute; Fiorini, Emma; Benedito, Rui; Besseyrias, Valerie; Schuster; Gossler, Karin; Pierres, Michel; Manley, Nancy R; Duarte, Antonio; Macdonald, H Robson; Radtke, F. Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. *The Journal of experimental medicine* **205**, 2515–2523 (2008).

35. Hozumi, Katsuto; Mailhos, Carolina; Negishi, Naoko; Hirano, Ken-ichi; Yahata, Takashi; Ando, Kiyoshi; Zuklys, Saulius; Holländer, Georg a; Shima, David T; Habu, S. Delta-like 4 is indispensable in thymic environment specific for T cell development. *The Journal of experimental medicine* **205**, 2507–2513 (2008).

36. Robey, E; Chang, D; Itano, A; Cado, D; Alexander, H; Lans, D; Weinmaster, G; Salmon, P. An activated form of Notch influences the choice between CD4 and CD8 T cell lineages. *Cell* **87**, 483–492 (1996).

37. Washburn, T; Schweighoffer, E; Gridley, T; Chang, D; Fowlkes, B J; Cado, D; Robey, E. Notch activity influences the alphabeta versus gammadelta T cell lineage decision. (1997) Cell 88 (6) p. 833-843. *Cell* **88**, 833–843 (1997).

38. Jenkinson, E. J., Jenkinson, W. E., Rossi, S. W. & Anderson, G. The thymus and T-cell commitment: the right niche for Notch? *Nature reviews. Immunology* **6**, 551–555 (2006).

39. Parreira, L., Neves, H. & Simões, S. Notch and lymphopoiesis: a view from the microenvironment. *Seminars in Immunology* **15**, 81–89 (2003).

40. Masuda, K. *et al.* Notch activation in thymic epithelial cells induces development of thymic microenvironments. *Molecular immunology* **46**, 1756–1767 (2009).

41. Lipinski, M., Braham, K., Caillaud, J., Carlu, C. & Tursz, T. HNK-1 antibody detects an antigen expressed on neuroectodermal cells. *The Journal of experimental medicine* **158**, 1775–1780 (1983).

42. Harman, B. & Jenkinson, W. T/B lineage choice occurs prior to intrathymic Notch signaling. *Blood* **106**, 886–892 (2005).

43. Coltey, M., Bucy, R. & Chen, C. Analysis of the first two waves of thymus homing stem cells and their T cell progeny in chick-quail chimeras. *The Journal of experimental medicine* **170**, 543–557 (1989).

44. Coltey, M., Jotereau, F. & Douarin, N. L. Evidence for a cyclic renewal of lymphocyte precursor cells in the embryonic chick thymus. *Cell differentiation* **22,** 71-82 (1987).

45. Griffith, Ann V; Fallahi, Mohammad; Nakase, Hiroshi; Gosink, Mark; Young, Brandon; Petrie, H. T. Spatial mapping of thymic stromal microenvironments reveals unique features influencing T lymphoid differentiation. *Immunity* **31**, 999–1009 (2009).

APPENDIX I

BUFFERS, MEDIA AND OTHER SOLUTIONS

BUFFERS FOR MULTIPLE USES

TAE 1X	
EDTA (pH 8)	1mM
Acetic acid	20mM
Tris base	40mM

PBS 1X	
NaCl	137mM
KCL	2.7mM
KCL	2.7mM
Na_2HPO_4	10mM
KH_2PO_4	2mM
Adjust pH to 7.4 with HCl	

PBT 0.1%	
Tween 20	0.1%
PBS	to final volume

BACTERIAL GROWTH

Lysogeny Broth (LB) medium	
Tryptone	1%
Yeast extract	0.5%
NaCl	1%

LB agar
7.5 g agar per 500 mL of LB medium

SOLUTIONS FOR *IN SITU* HYBRIDIZATION

3.7% Paraformaldehyde (PFA) in PBS	
7.4% Paraformaldehyde (stock)	1:2
PBS (1X)	1:2

Hybridization Solution	
100% Formamide (deionized)	50%
50% Dextran sulfate	10%
50X Denhardt's (Rnase free)	1%
10 mg/ml Yeast tRNA	1mg/mL
Salt mix 10X	1X
H_2O	to final volume

Washing Solution

100% Formamide (deionized)	50%
20X SSC pH7.5	1X
Tween 20	0.1%
H_2O	to final volume

MAB pH=7,5

2M Maleic Acid	0.1M
5M NaCl	0.15M
10N NaOH	1N
H_2O	to final volume

Adjust pH to 7.5 with NaOH 10M

MABT

Tween 20	0.1%
MAB	to final volume

NTM pH=9.5

1M Tris pH=9.5	0.1M
5M NaCl	0.1M
2M $MgCl_2$	50mM
H_2O	to final volume

SOLUTIONS FOR FLOW CYTOMETRY

MEFs medium

FBS	10%
100X Pen Strep	1X
Glutamine	1%
DMEM	to final volume

MACS Buffer

BSA	0,5%
EDTA	2mM
PBS 1X	to final volume

APPENDIX II

PROTOCOLS

PHENOL:CHLOROFORM EXTRACTION AND PRECIPITATION WITH ETHANOL

This step was performed to remove proteins from a nucleic acid solution. 150µL of phenol:chloroform 1:1 (v/v) (Ambion) were added per sample and the lower phase (chloroform) was removed by pipetting with thin tips. After centrifuging at 11000rpm for 5 min at 4ºC, the upper aqueous phase, which contains nucleic acids, was recovered. Again, 150µL of phenol:chloroform 1:1 (v/v) were added per sample to the aqueous phase, centrifuged at 11000rpm for 10 min at 4ºC and then the upper phase recovered. Precipitation of the DNA was done by adding 15µL of sodium acetate (NaAC) 3M and 450µL of absolute ethanol per sample and incubating at -80ºC for 30 min. The mixture was then centrifuged at 13000rpm for 30 min at 4ºC. The supernatant was removed, the pellet dried and ressuspended in 10µL of RNase-free water.

FLOW CYTOMETRY PROTOCOLS

THYMOCYTES SUSPENSION PREPARATION

1. Isolate thymus lobes and maintain them overnight at 4ºC in PBS (1X)/ 2% FBS/ Penstrep (1X).
2. Mechanically disrupt thymus lobes by gently pressing the tissue through a nylon mesh (Cell strainer 70µm BD Falcon).
3. Wash with PBS (1X)/ 2% FBS/ Penstrep (1X), and centrifuge at 1800rpm for 10 min.
4. Ressuspend thymocytes in 6mL of MEFs medium and add it to 3mL of Ficoll-Paque PLUS (GE Healthcare Life Sciences) without mixing the two phases. Centrifuge at 1800rpm for 30 min.
5. Recover the white phase between Ficoll and MEFs medium, and ressuspend it in MEFs medium. Centrifuge at 1400rpm for 10 min, to wash the cells.
6. Ressuspend the cells in MEFs medium, and count them. Use 10^5 cells for each staining condition.

STAINING

7. Wash with 100µL of MACs buffer. Centrifuge at 1700rpm for 7 min.

8. Add 100µL of antibody(ies) dilutions in MACs Buffer. Incubate for 30 min at 4ºC in the dark.

9. Add 100µL of MACs Buffer. Centrifuge at 1700rpm for 7 min.

10. Ressuspend in MACs Buffer.

APPENDIX III

FLOW CYTOMETRY RESULTS

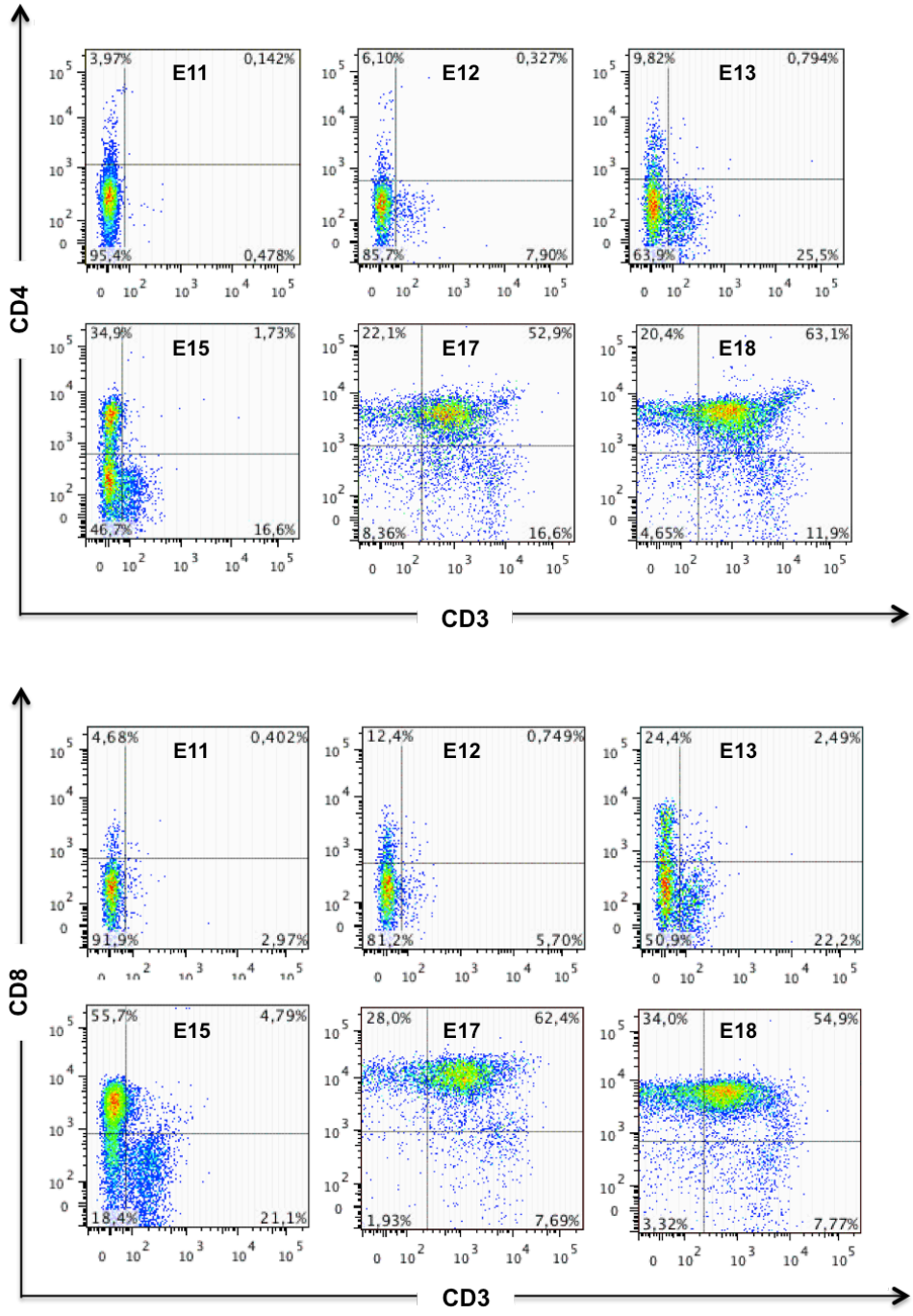

Figure 11. Percentages of thymocytes expressing the CD3, CD4 and CD8 markers during chicken thymus embryonic development.